# Cross-talk between interleukin 1$\beta$ (IL-1$\beta$) and IL-6 signalling pathways: IL-1$\beta$ selectively inhibits IL-6-activated signal transducer and activator of transcription factor 1 (STAT1) by a proteasome-dependent mechanism

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Interleukin 1$\beta$ (IL-1$\beta$) suppresses the IL-6-dependent induction of type II acute-phase response genes, but the underlying mechanism for this suppression remains uncertain. Here we report that treatment of human hepatocullular carcinoma HepG2 cells with IL-1$\beta$ inhibited the IL-6-dependent binding of signal transducer and activator of transcription factor (STAT)1, but not that of STAT3, to the high-affinity serum-inducible element ('SIE'). Furthermore, IL-1$\beta$ selectively down-regulated the IL-6-induced tyrosine phosphorylation of STAT1 without affecting the level of STAT1 or tyrosine phosphorylation of STAT3. Kinase assays *in vitro* indicated that the inhibition of STAT1 phosphorylation by IL-1$\beta$ was not due to an upstream blockade of Janus kinase (JAK1 or JAK2) activation. However, pretreatment with the proteasome inhibitor MG132 under conditions that prevented the IL-1$\beta$-dependent activation of the nuclear factor NF-$\kappa$B also blocked the inhibitory effect of IL-1$\beta$ on IL-6-activated STAT1. In related experiments, the protein tyrosine phosphatase inhibitor $Na_3VO_4$ also antagonized the inhibitory effect of IL-1$\beta$ on the activation of STAT1 by IL-6. Taken together, these findings indicate that, by using a proteasome-dependent mechanism, IL-1$\beta$ concomitantly induces NF-$\kappa$B activation and dephosphorylates IL-6-activated STAT1; the latter might partly account for the inhibition by IL-1$\beta$ of the IL-6-dependent induction of type II acute-phase genes.

Key words: acute-phase protein, Janus kinase, nuclear factor $\kappa$B, liver cells, protein tyrosine phosphatase.

## INTRODUCTION

The acute-phase reaction that occurs in response to tissue injury and infection is mediated by hepatic synthesis of distinct classes of acute-phase proteins [1–4]. Expression of these acute-phase proteins is regulated predominantly by the actions of interleukin 6 (IL-6) and IL-1. Consequently, the acute-phase proteins are classified into type I and type II subgroups. Type I acute-phase proteins (e.g. C-reactive protein, serum amyloid A and $\alpha_1$ acid glycoprotein) can be maximally induced by the synergistic action of IL-6 and IL-1, whereas type II proteins (e.g. several fibrinogen chains and $\alpha_2$-macroglobulin) can be maximally induced by IL-6 alone. In fact, with type II genes, IL-1 acts to suppress rather than enhance the IL-6-dependent induction of gene expression [1–4]. However, the molecular mechanism by which IL-1 down-regulates the IL-6-induced expression of acute-phase proteins (including fibrinogen) remains uncertain.

Presumably, the inhibitory action of IL-1 on IL-6-inducible gene expression depends on interruption of the IL-6 signal-transduction pathway from the cell surface to the nucleus. Thus IL-6 initiates its actions in the liver by interaction with the gp130 signalling protein [5]. The interaction of IL-6 with the $\alpha$-chains of the IL-6 receptor (IL-6R$\alpha$) induces the homodimerization of gp130 and the consequent activation of three receptor-associated Janus kinases (JAKs): JAK1, JAK2 and Tyk2 [5–7]. The activated IL-6R$\alpha$–gp130–JAK complex recruits and phosphorylates the signal transducer and activator of transcription (STAT) family members designated STAT1 and STAT3, which then form homodimers or heterodimers that translocate to the nucleus to activate the transcription of many target genes, including acute-phase response genes [5–7]. Transcriptional activation by STAT proteins can be terminated by the action of protein tyrosine phosphatases (PTPs) [8–17], by degradation by the ubiquitin–proteasome pathway [18–21], by the action of inhibitory molecules such as SOCS/JAB/SSI/CIS (reviewed in [22]) or by the action of several protein kinases, including p42/44 mitogen-activated protein (MAP) kinase [23–25], but the role of these steps in IL-6-dependent gene expression is uncertain. In addition to the JAK–STAT signalling pathway, IL-6 also activates phosphoinositide 3-kinase and weakly stimulates Ras/MAP kinase in the liver [26,27].

In attempting to define which IL-6-driven signalling steps are targets for inhibition by IL-1, we also reviewed the actions of IL-1 on gene expression. Thus IL-1$\beta$ exerts its biological effects (in the liver and other organs) through initial interaction with the IL-1 type I receptor [28]. The receptor binding of IL-1$\beta$ leads to the recruitment of two IL-1-receptor-associated kinases, IRAK-1 and IRAK-2, and the subsequent activation of NF-$\kappa$B-inducing kinase (NIK) and then I$\kappa$B kinase. The activated I$\kappa$B kinase acts to phosphorylate the NF-$\kappa$B inhibitory protein I$\kappa$B$\alpha$ at $Ser^{32}$ and $Ser^{36}$, leading to its ubiquitination and degradation through the ubiquitin-dependent proteasome pathway [28–34]. The degradation of I$\kappa$B allows NF-$\kappa$B to translocate to the nucleus and mediate the more efficient transcription of appropriate target genes containing the NF-$\kappa$B-binding site [28–34]. In addition to the NF-$\kappa$B signalling pathway, IL-1$\beta$ also activates p42/44 MAP

Abbreviations used: EMSA, electrophoretic gel-mobility shift assay, IFN, interferon; IL, interleukin; JAK, Janus kinase; MAP, mitogen-activated protein; NIK, NF-$\kappa$B-inducing kinase; PTP, protein tyrosine phosphatase; STAT, signal transducer and activator of transcription.

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kinase, p38 MAP kinase, c-Jun N-terminal kinase (JNK) and phosphoinositide 3-kinase [28–34].

In the present study we used a hepatic cell model system to analyse the cross-talk between IL-1$\beta$ and IL-6 signalling. We show that IL-1$\beta$ exerts its inhibitory action in this system by selectively down-regulating the phosphorylation of STAT1. Additional experiments indicate that the IL-1$\beta$-dependent down-regulation of STAT1 phosphorylation relies on a proteasome-dependent pathway that might be analogous to the IL-1$\beta$-dependent degradation of I$\kappa$B. We also present evidence that the capacity of IL-1$\beta$ to increase the ratio of unphosphorylated to phosphorylated STAT1 (and so inactivate STAT1) might depend on increases in phosphatase activity. We reconcile and integrate each of these findings with a new model that allows for cross-talk between IL-1$\beta$ and IL-6 signalling.

## MATERIALS AND METHODS

### Materials

Antibodies against STAT1, STAT3, JAK1 and JAK2 were purchased from Upstate Biotechnology (Lake Placid, NY, U.S.A.). Anti-[phosphotyrosine-STAT3 (Tyr$^{705}$)] and anti-[phosphotyrosine-STAT1 (Tyr$^{701}$)] antibodies were obtained from Bio-Lab (Beverly, MA, U.S.A.). MG132 was purchased from Calbiochem (San Diego, CA, U.S.A.). [$\gamma$-$^{32}$P]ATP was obtained from DuPont NEN (Boston, MA, U.S.A.). The following reagents were obtained from Sigma Chemicals (St Louis, MO, U.S.A.): $Na_3VO_4$, Nonidet P40, IL-6 and IL-1$\beta$. The concentration of IL-6 used in this paper was 20 ng/ml. The HepG2 cell line was obtained from American Type Culture Collection (Manassas, VA, U.S.A.) and cultured as directed.

### Electrophoretic gel-mobility shift assay (EMSA)

The EMSA for STAT binding was performed as described previously [35,36]. In brief, DNA mobility-shift assays were performed in 20 $\mu$l volumes with 20 mM Tris/HCl (pH 7.9)/1.5 % (v/v) glycerol/50 $\mu$g/ml BSA/1 mM dithiothreitol/0.5 mM PMSF containing 2 $\mu$g of poly(dI-dC), 1 ng of $^{32}$P-labelled probe and 10 $\mu$g of nuclear extract. Reactions were incubated at 25 °C for 20 min and subsequently analysed by non-denaturing PAGE [10 % (w/v) gel] in 0.5 × Tris/borate/EDTA buffer, consisting of 44.5 mM Tris/HCl, pH 8.2, 44.5 mM boric acid and 1 mM EDTA. After the gel had been pre-run at 100 V for 2 h, electrophoresis was performed at 270 V for 2 h at 4 °C. The gels were exposed to a PhosphorImager™ Exposure Cassette and analysed with the PhosphorImager™ ImageQuant™ program (Molecular Dynamics). The STAT-binding site in the double-stranded oligonucleotide m67 [the high-affinity serum-inducible element (SIE)], consisting of 5′-GTC GAC ATT TCC CGT AAA TCG TCG A-3′ [37,38], and the NF-$\kappa$B-binding site in the double-stranded oligonucleotide 5′-AGT TGA GGG GAC TTT CCC AGG-3′, were used as probes to determine STAT1/STAT3 and NF-$\kappa$B binding respectively.

### Cell extraction, SDS/PAGE and Western blotting

Cells were lysed in lysis buffer [30 mM Tris/HCl (pH 7.5)/150 mM NaCl/1 mM PMSF/1 mM $Na_3VO_4$/1 % (v/v) Nonidet P40/10 % (v/v) glycerol] for 15 min at 4 °C and vortex-mixed, then centrifuged at 3100 ***g*** and 4 °C for 10 min. The supernatants were mixed in Laemmli running buffer, boiled for 4 min and then subjected to SDS/PAGE. After electrophoresis, proteins were transferred on nitrocellulose membranes and blotted against anti-STAT antibodies. Membranes were washed with TPBS buffer [0.05 % (v/v) Tween 20 in PBS (pH 7.4)] and incubated with a 1:4000 dilution of horseradish-peroxidase-conjugated secondary antibodies for 45 min. Protein bands were detected by an enhanced chemiluminescence reaction (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.).

### JAK kinase assay

To assess JAK phosphorylation, cells were washed twice with PBS, pH 7.4, containing 1 mM $Na_3VO_4$ and lysed in 0.5 ml of lysis buffer. The total cell extracts were immunoprecipitated with anti-JAK1 or anti-JAK2 antibodies, washed twice with lysis buffer and then once with kinase buffer [50 mM Tris/HCl (pH 7.4)/5 mM $MgCl_2$/10 mM $MnCl_2$/0.1 mM $Na_3VO_4$]. Pellets were resuspended in 50 $\mu$l of kinase buffer containing 5 $\mu$Ci of [$\gamma^{32}$P]ATP and incubated at 30 °C for 10 min. Beads were washed twice with 500 $\mu$l of stop buffer [50 mM Tris/HCl (pH 7.4)/150 mM NaCl/10 mM EDTA], then boiled in SDS sample buffer containing 2.5 % (v/v) 2-mercaptoethanol for 5 min. The solubilized proteins were resolved by SDS/PAGE and quantified by PhosphorImaging.

### Expression of dominant-negative mutants

The dominant-negative mutants of NIK were transfected into the cells by an adenovirus-lysine-mediated procedure as described previously [39]. This method can achieve approx. 80 % transfection efficiency. In brief, adenovirus-DNA complexes were prepared by incubating lysine-modified adenovirus with dominant-negative mutants of NIK for 30 min at 25 °C in the dark, followed by a 30 min incubation with polylysine at a molar concentration equivalent to 125-fold the molar plasmid DNA concentrations. Adenovirus-DNA-lysine complex was then added to the cells and incubated for 8 h at 37 °C. The cells were washed with medium to remove virus and cultured for an additional 48 h in Dulbecco's modified Eagle's medium containing 10 % (v/v) fetal-calf serum. The dominant-negative mutants of NIK (KK429-430AA) were generous gifts from Dr David Wallach (Weizmann Institute of Science, Rehovot, Israel) as described previously [40].

## RESULTS

### IL-1$\beta$ rapidly down-regulates IL-6-induced STAT1 but not STAT3 activation

To determine the mechanism by which IL-1$\beta$ inhibits IL-6-induced type II acute-phase gene expression, the effect of IL-1$\beta$ on the IL-6 signalling pathway was examined. As shown in Figure 1(A), exposure of HepG2 cells to IL-6 for 30 min activated STAT1/STAT3, as indicated by the binding of STAT1/STAT3 to its consensus site in the m67 oligonucleotide probe (Figure 1A, lane 2 compared with lane 1). The identities of STAT1 and STAT3 were confirmed by the gel-mobility supershift assay with anti-STAT1 and anti-STAT3 antibodies (Figure 1B, left panel). The DNA binding of STAT1/STAT3 was markedly inhibited by a 30 min pretreatment with IL-1$\beta$, with inhibition already evident at 0.1 ng/ml. Interestingly, IL-1$\beta$ treatment even at a high concentration (100 ng/ml) was unable to suppress completely the IL-6-induced STAT1/STAT3 binding to the m67 probe (Figure 1A), suggesting that IL-1 only partly inhibits the formation of the IL-6-induced STAT-containing complexes or selectively inhibits a part of this complex.

To determine better whether a part of the IL-6-induced STAT complex was inhibited by IL-1$\beta$, gel mobility-shift assays were performed in the absence and the presence of anti-STAT1 and anti-STAT3 antibodies. The IL-6-inducible STAT-binding complex interacted with both anti-STAT1 and anti-STAT3 antibodies

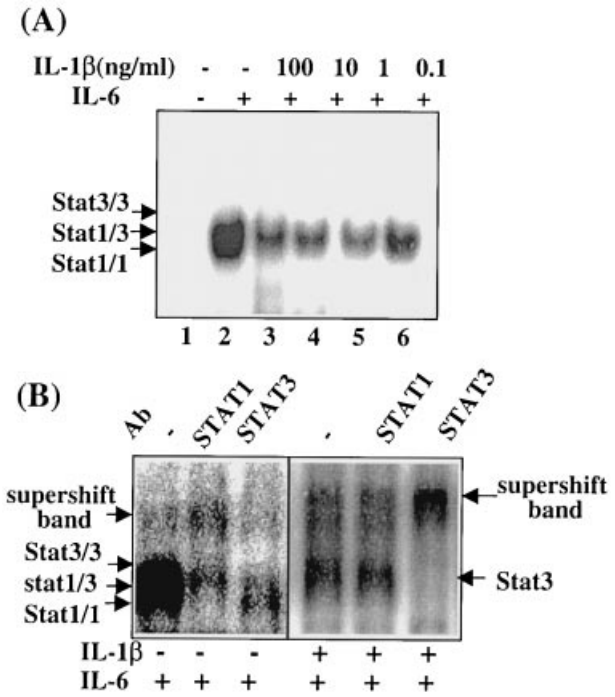



**Figure 1 IL-1$\beta$ rapidly suppresses IL-6-activated STAT1 but not STAT3**

(**A**) HepG2 cells were treated for 30 min with various concentrations of IL-1$\beta$ as indicated, followed by a 30 min stimulation with IL-6. Cell extracts were subjected to EMSA with m67 as an oligonucleotide probe. (**B**) Cell extracts from HepG2 cells treated with IL-6 or with IL-1$\beta$ plus IL-6 were incubated with various antibodies as indicated and then subjected to EMSA with the m67 probe. An autoradiogram representative of three independent experiments is shown.

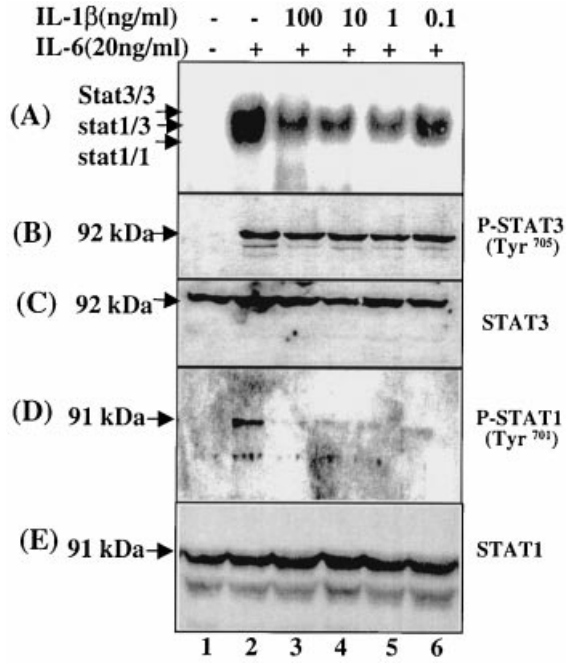



**Figure 2 IL-1$\beta$ inhibits the IL-6-induced tyrosine phosphorylation of STAT1 but not that of STAT3**

HepG2 cells were incubated for 30 min with various concentrations of IL-1$\beta$, followed by a 30 min stimulation with IL-6. Cell lysates were then subjected to EMSA with the m67 oligonucleotide as a probe (**A**), or to Western-blot analysis with anti-[phosphotyrosine-STAT3 (Tyr$^{705}$)] (**B**), anti-STAT3 (**C**), anti-[phosphotyrosine-STAT1 (Tyr$^{701}$)] (**D**) or anti-STAT1 (**E**) antibodies. Blots shown are representative of three independent experiments.

(Figure 1B, left panel) but not with control anti-STAT5 antibody (results not shown). As noted above, these findings indicate that IL-6 was able to activate both STAT1 and STAT3 selectively in HepG2 cells. Interestingly, however, after treatment with IL-1$\beta$ plus IL-6, the resulting STAT complex interacted with anti-STAT3 antibody, but no longer interacted with anti-STAT1 antibody (Figure 1B, right panel). This finding indicated that STAT1 was no longer capable of binding to its regulatory site after IL-1$\beta$ treatment, and therefore further suggested that IL-1$\beta$ selectively down-regulated IL-6-dependent signalling at the level of STAT1 activation status.

### IL-1$\beta$ inhibits IL-6-induced STAT1 tyrosine phosphorylation, but not STAT1 expression or STAT3 tyrosine phosphorylation

The capacity of IL-1$\beta$ to inhibit IL-6-induced STAT1 (or STAT3) binding to its regulatory site could be due to the down-regulation of tyrosine phosphorylation. To examine this possibility more directly, Western blot analysis was performed with anti-(phosphotyrosine-STAT1) and anti-(phosphotyrosine-STAT3) antibodies. Results indicated that treatment with IL-6 induced significant STAT3 tyrosine phosphorylation (Figure 2B, lane 2); pretreatment of HepG2 cells with IL-1$\beta$ for 30 min did not alter this phosphorylation level (Figure 2B, lanes 3–6) or the cellular level of STAT3 protein (Figure 2C). In control experiments we found that treatment with IL-1$\beta$ alone did not alter the basal level of STAT3 tyrosine phosphorylation (results not shown). In contrast with STAT3 phosphorylation, however, STAT1 tyrosine phosphorylation induced by IL-6 was markedly suppressed by treatment with IL-1$\beta$ (Figure 2D); inhibition of phosphorylation

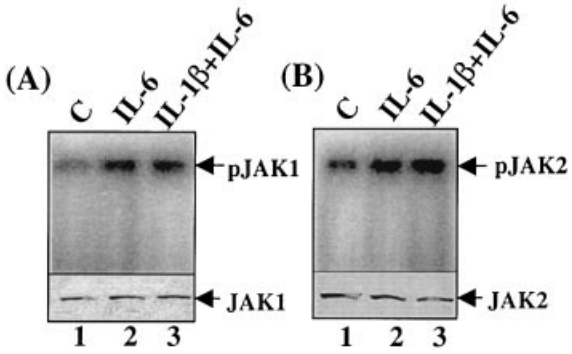



**Figure 3 Effects of IL-1$\beta$ on the IL-6-induced phosphorylation of receptor-associated kinases (JAK1 and JAK2)**

HepG2 cells were treated with IL-1$\beta$ (10 ng/ml) for 30 min, followed by a 5 min stimulation with IL-6. Cell extracts were immunoprecipitated with anti-JAK1 (**A**) or anti-JAK2 (**B**) antibodies. The immunoprecipitated JAK1 or JAK2 proteins were subjected to kinase assays *in vitro* as described in the Materials and methods section. Autoradiograms shown are representative of two independent experiments.

was accomplished without a change in the cellular levels of STAT1 protein (Figure 2E). Taken together, these findings indicate that IL-1$\beta$ inhibits IL-6-induced STAT1 but not STAT3 tyrosine phosphorylation. This finding is consistent with the suppression by IL-1$\beta$ of the IL-6-activated binding of STAT1, but not that of STAT3, to its consensus site, as detected above by gel mobility-shift assay (Figure 1).

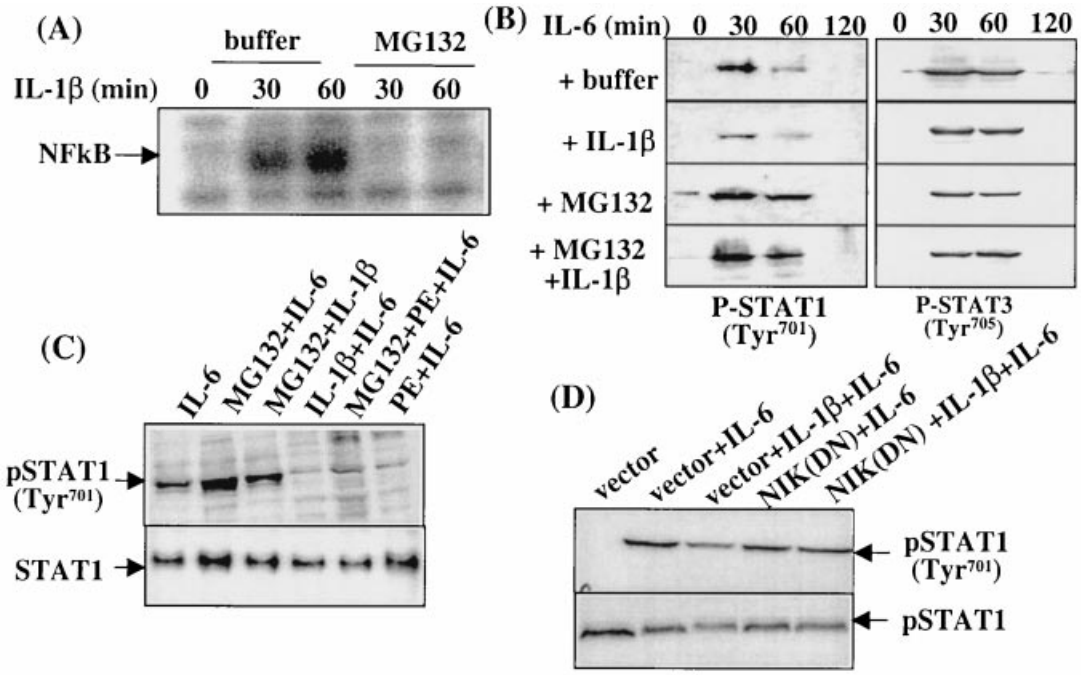



**Figure 4 Proteasome inhibitor MG132 attenuates the stimulation by IL-1$\beta$ of the NF-$\kappa$B and IL-1$\beta$ suppression of the IL-6-activated tyrosine phosphorylation of STAT1**

(**A**) HepG2 cells were treated with buffer or MG132 (50 $\mu$M) for 30 min, then stimulated with IL-1$\beta$ (10 ng/ml) for the indicated periods. Cell extracts were then subjected to EMSA by using an oligonucleotide with an NF-$\kappa$B site as a probe. (**B**) HepG2 cells were incubated with or without MG132 (50 $\mu$M) for 30 min, then treated with or without IL-1$\beta$ (10 ng/ml) for 30 min, followed by stimulation with IL-6 for the indicated periods. (**C**) TFG2 cells (transfected HepG2 cells with $\alpha_{1B}$ adrenergic receptor [27]) were incubated with MG132 (50 $\mu$M) for 30 min, then stimulated with IL-1$\beta$ or phenylephrine (PE, 10 $\mu$M) for 30 min, followed by a 30 min stimulation with IL-6. (**D**) HepG2 cells were transfected with control vector or dominant-negative (DN) NIK mutants for 48 h, then treated with IL-1$\beta$ for 30 min, followed by a 30 min stimulation with IL-6. In (**B**), (**C**) and (**D**), cell extracts were subjected to Western-blot analysis with anti-[phosphotyrosine-STAT1 (Tyr$^{701}$)] or anti-[phosphotyrosine-STAT3 (Tyr$^{705}$)] antibodies as indicated. Autoradiograms or blots shown are representative of three separate experiments.

### IL-1$\beta$ does not significantly inhibit IL-6-induced JAK phosphorylation

To test whether IL-6-induced STAT1 tyrosine phosphorylation was inhibited by IL-1$\beta$ action at an upstream site, JAK1 and JAK2 phosphorylation was analysed with a kinase assay *in vitro*. In these experiments, treatment with IL-6 for 5 min induced a marked increase in the levels of JAK1 and JAK2 phosphorylation in cellular extracts prepared from HepG2 cells (Figure 3, lanes 2). However, neither JAK1 nor JAK2 phosphorylation was significantly affected by treatment with IL-1$\beta$ (Figure 3). In control experiments we found that treatment with IL-1$\beta$ alone did not affect the basal levels of JAK1 or JAK2 phosphorylation (results not shown). These results indicate that IL-1$\beta$ does not significantly suppress the IL-6-induced phosphorylation of either JAK1 or JAK2 tyrosine kinases. The findings (along with the absence of a change in STAT3 phosphorylation) suggested that the down-regulation of STAT1 activation by IL-1$\beta$ might occur at a site further downstream in the IL-6-driven JAK–STAT signalling pathway, i.e. at the level of STAT1 dephosphorylation.

### IL-1$\beta$ inhibits IL-6-induced activation of STAT1 activation by a proteasome-dependent pathway

Previous reports have provided a role for the ubiquitin–proteasome pathway in IL-1$\beta$ activation of NF-$\kappa$B [29–31]. We therefore questioned whether this type of pathway might also be involved in the inhibition by IL-1$\beta$ of the activation of STAT1 by IL-6. To test this possibility, the highly selective proteasome inhibitor MG132 was used. As shown in Figure 4(A), IL-1$\beta$ rapidly induced NF-$\kappa$B activation (on the basis of DNA binding); this activation was markedly suppressed by pretreatment with MG132. This finding is consistent with the role of the ubiquitin–proteasome pathway in degrading an inhibitor (I$\kappa$B), thus allowing the IL-1$\beta$-driven activation of NF-$\kappa$B [29–31]. Additional experiments indicated that the pretreatment of HepG2 cells with MG132 significantly enhanced and prolonged the IL-6-induced tyrosine phosphorylation of STAT1, but not that of STAT3 (Figure 4B). Treatment of HepG2 cells with IL-1$\beta$ and/or IL-6 and/or MG132 did not affect the levels of STAT1 protein expression (results not shown). This finding suggested that the ubiquitin–proteasome pathway was also involved in degrading a target that normally mediated up-regulation of the activation of STAT1 by IL-6 (Figure 4B). Furthermore, pretreatment of HepG2 cells with MG132 markedly antagonized the expected IL-1$\beta$-dependent inhibition of the activation of STAT1 by IL-6 (Figure 4B). Because MG132 markedly potentiated IL-6-activated STAT1, MG132's reversal of the inhibition by IL-1$\beta$ of IL-6-activated STAT1 could be due to direct or non-specific stabilization of STAT1 phosphorylation. However, this seems unlikely, because MG132 did not antagonize the phenylephrine-dependent inhibition of IL-6-activated STAT1, as demonstrated in Figure 4(C). Treatment of HepG2 cells with IL-1$\beta$ and/or IL-6 and/or phenylephrine and/or MG132 did not affect the levels of STAT1 protein expression (Figure 4C, bottom panel). These findings suggest that the proteasome pathway is crucial for the suppression of IL-6-activated STAT1 by IL-1$\beta$.

To confirm the involvement of NIK-driven proteasome pathway in the suppression of IL-6-activated STAT1 by IL-1, NIK

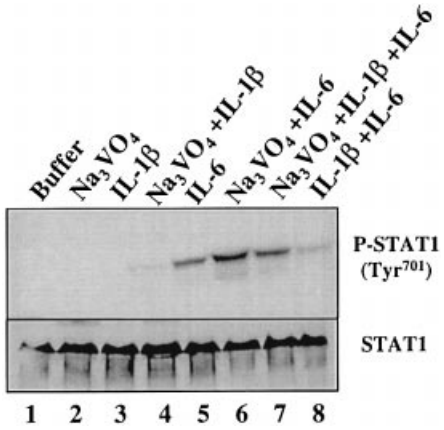
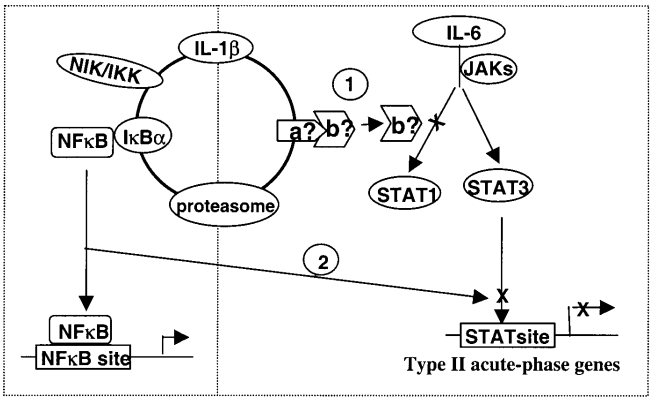



**Figure 5 Evidence for the involvement of tyrosine phosphatases in the inhibition of IL-6-activated STAT1 by IL-1$\beta$**

HepG2 cells were incubated with $Na_3VO_4$ (0.2 mM) for 30 min, then treated with IL-1$\beta$ (10 ng/ml) for 30 min, followed by a 30 min stimulation with IL-6. The cell extracts were then subjected to Western blotting with anti-[phosphotyrosine-STAT1 ($Tyr^{701}$)] antibody (top panel) and anti-STAT1 antibody (bottom panel). Blots shown are representative of three independent experiments.




**Scheme 1 Model for cross-talk between the IL-1$\beta$ and IL-6 signal-transduction pathways to the nucleus and for the regulation of type II acute-phase response genes**

For IL-1$\beta$-dependent gene expression (left panel), IL-1$\beta$ binds to its receptor and activates NIK, followed by the activation of I$\kappa$B kinase (IKK), which mediates the subsequent phosphorylation of I$\kappa$B and the degradation of I$\kappa$B via the ubiquitin–proteasome pathway. These steps allow for the binding of NF-$\kappa$B to its DNA site and for the transcription of NF-$\kappa$B target genes. For the IL-1$\beta$-dependent inhibition of the expression of IL-6-induced type II acute-phase genes (right panel), two mechanisms might be involved: (1) IL-1$\beta$ might similarly act to promote the proteasome-dependent degradation of an unknown protein (a?), followed by the release of an inhibitory factor (b?) that attenuates STAT1 phosphorylation; or (2) activated NF-$\kappa$B might compete with STAT binding and consequently inhibit IL-6-activated STAT binding on type II acute-phase gene promoters [43]. See the text for details.

(KK429-430AA) dominant-negative expression vectors were used. As shown in Figure 4(D), overexpression of NIK (KK429-430AA) did not significantly affect IL-6-activated STAT1, but markedly antagonized the inhibitory action of IL-1$\beta$. Transfection with the dominant-negative mutants of NIK did not affect the levels of STAT1 protein expression (Figure 4D, bottom panel). Taken together, these findings suggest (as developed below) that the proteasome-dependent degradation of a protein that potentiates STAT1 activation, i.e. the degradation of an inhibitor of STAT1 tyrosine phosphatase, might occur in a manner analogous to degradation of the I$\kappa$B inhibitor of NF-$\kappa$B activation.

### One or more $Na_3VO_4$-sensitive PTPs are involved in the inhibition by IL-1$\beta$ of IL-6-induced STAT1 activation

As noted above, dephosphorylation of the JAK–STAT proteins by PTPs is another important mechanism for the down-regulation of this signalling pathway [8–17]. To define more directly the role for PTPs in IL-1$\beta$-dependent inhibition of IL-6-activated STAT1, we tested the effect of the PTP inhibitor $Na_3VO_4$ on the suppression by IL-1$\beta$ of IL-6-activated STAT1 in HepG2 cells. In these experiments, cells were treated with IL-6 alone and IL-6 plus IL-1$\beta$ for 30 min, without or with pretreatment with $Na_3VO_4$ for 30 min. Pretreatment with $Na_3VO_4$ significantly enhanced the induction by IL-6 of STAT1 tyrosine phosphorylation and markedly antagonized the inhibition by that induction (Figure 5). These findings suggested again that IL-1$\beta$ exerts its inhibitory effect on the activation of STAT1 by IL-6 by influencing the dephosphorylation of STAT1 by PTPs.

## DISCUSSION

This report presents the first mechanistic evidence for cross-talk between the IL-1$\beta$ and IL-6 signalling pathways. In particular, we show that IL-1$\beta$ exerts its inhibitory action on the IL-6-dependent expression of type II acute-phase proteins by selectively down-regulating the phosphorylation of STAT1. Impaired activation of STAT1 occurs in the absence of upstream alterations in IL-6-receptor–gp130-associated kinases (JAK) activities, implying that the inactivation of STAT1 occurs at downstream step, i.e. the dephosphorylation of STAT1 by a PTP [8–17]. In fact, we also present initial evidence that the capacity of IL-1$\beta$ to increase the ratio of unphosphorylated to phosphorylated STAT1 (and so to inactivate STAT1) might depend on increases in phosphatase activity, because the effect is negated by a phosphatase inhibitor ($Na_3VO_4$). Additional experiments indicate that the IL-1$\beta$-dependent down-regulation of STAT1 phosphorylation relies on a proteasome-dependent pathway, because this effect is antagonized by a proteasome inhibitor (MG132). We therefore conclude that proteasome-dependent degradation of a putative inhibitor of a STAT1 PTP would best reconcile all of these findings. We have integrated each of these findings in a new model (summarized in Scheme 1) that allows for cross-talk between IL-1$\beta$ and IL-6 signalling and consequently the selective expression of acute-phase target genes. The rationale for this model is presented in the following discussion.

In our model in Scheme 1 for type II acute-phase gene control, we note that IL-1$\beta$ acts to initiate NF-$\kappa$B-dependent gene expression while it concomitantly down-regulates IL-6-dependent gene expression. In IL-1$\beta$-induced NF-$\kappa$B-dependent gene expression, IL-1 binding to the IL-1 receptor initiates the rapid and sequential activation of NIK and then I$\kappa$B kinase, which phosphorylates the NF-$\kappa$B inhibitory protein I$\kappa$B$\alpha$ and so enables the subsequent degradation of I$\kappa$B through the ubiquitin–proteasome pathway [28–34]. The loss of I$\kappa$B inhibition then permits the translocation of NF-$\kappa$B to nuclear sites for DNA binding and target gene expression (Scheme 1, left panel).

As noted above, we propose an analogous set of events for the IL-1$\beta$-dependent inhibition of IL-6-induced STAT1/STAT3-dependent gene expression. In this case, IL-1$\beta$ might also drive degradation, but here it drives the degradation of an unknown protein (a?), followed by the release of an inhibitory factor (b?) that attenuates STAT1 tyrosine phosphorylation and so blocks

the STAT1-dependent activation of transcription, including expression of the gene for $\gamma$ fibrinogen (Scheme 1). The role of a ubiquitin–proteasome-dependent pathway in these events was further supported by our finding that the proteasome inhibitor MG132 simultaneously blocked the capacities of IL-1$\beta$ to activate NF-$\kappa$B and to inactivate STAT1. Because MG132 itself can significantly increase the level of IL-6-activated STAT1, the drug's reversal of the inhibition by IL-1$\beta$ of IL-6-activated STAT1 could be due to direct and non-specific stabilization of STAT1 phosphorylation. However, this appears unlikely, because MG132 did not reverse the phenylephrine-dependent inhibition of IL-6-activated STAT1 (Figure 4C) or the ethanol-dependent inhibition of interferon (IFN)-$\gamma$-activated STAT1 [41].

The proteasome-dependent pathway for protein degradation has been implicated in the down-regulation of STAT activation induced by several other cytokines (besides IL-1 and IL-6). Thus, others have found that this pathway might down-regulate the IFN-$\gamma$-dependent or IL-3-dependent activation of STAT1 [18,20] and the activation of STAT5 by IL-2 or growth hormone [19,21]. These findings led us to speculate that IL-1$\beta$ might also suppress the activation of JAK–STAT signalling induced by these cytokines. Indeed, we have found that IL-1$\beta$ also markedly decreased both the IFN-$\alpha$-dependent and IFN-$\beta$-dependent activation of STAT1 in HepG2 cells (X. Shen, Z. Tian and B. Gao, unpublished work).

It has been reported that the proteasome-mediated inactivation of the JAK–STAT signalling pathway is due to the degradation of active STAT protein [18] or to a blockade of upstream signalling [19–21]. For example, IFN-$\gamma$-activated STAT1 can be ubiquitinated and degraded by the 26 S proteasome [18]. However, two lines of evidence indicate that the inhibition of IL-6-activated STAT1 by IL-1$\beta$ is not due to the direct ubiquitination and degradation of STAT1: (1) the total cellular STAT1 protein level was unchanged after treatment with IL-1$\beta$ (Figure 2E), and (2) no STAT1–ubiquitin conjugates were detected when immunoprecipitating STAT1 from IL-1$\beta$-treated and/or IL-6-treated cells (with or without MG132 treatment) and blotting the immune complex with anti-ubiquitin antibodies (X. Shen, Z. Tian and B. Gao, unpublished work). Others have also presented evidence that proteasome degradation might target upstream signalling steps (i.e. receptor-associated JAK) to down-regulate the JAK–STAT pathway [19–21]. However, our results indicate that IL-1$\beta$ does not inhibit the IL-6-dependent activation of JAK1 or JAK2 (Figure 3). Furthermore, IL-1$\beta$ does not suppress the IL-6-dependent activation of STAT3, a downstream target of JAK activities. Taken together, these findings suggest that the inhibition of IL-6-activated STAT1 by IL-1$\beta$ is not due to the suppression of JAK-dependent phosphorylation, and is more likely to be due to down-regulation of the IL-6-dependent activation of STAT1 at a downstream step, i.e. PTP-dependent dephosphorylation of STAT1.

A PTP-dependent mechanism for the down-regulation of the JAK–STAT signalling pathway has been implicated in several other model systems [8–17]. $Na_3VO_4$, the protein tyrosine inhibitor, markedly antagonized the suppression by IL-1$\beta$ of IL-6-activated STAT1 phosphorylation, suggesting that PTP might be involved. However, we cannot rule out the possibility that the reversal by $Na_3VO_4$ of the inhibition of IL-6-activated STAT1 by IL-1 could be due to direct and non-specific stabilization of STAT1 phosphorylation, because $Na_3VO_4$ significantly potentiated IL-6-activated STAT1, as shown in Figure 5. Candidate PTPs in other systems include SH2-domain-containing phosphatases such as SHP-1 or SHP-2 [8,13–16] and protein phosphatase 2A (PP2A) [17]. Our results show that IL-1$\beta$ selectively suppresses the IL-6-dependent phosphorylation of STAT1 but not that of STAT3, JAK1 or JAK2. These findings suggest that SHP-1, SHP-2 and PP2A are probably not involved in the suppression of IL-6-activated STAT1 by IL-1$\beta$, because SHP-1 and SHP-2 effectively dephosphorylate JAKs [8,13–16] and PP2A dephosphorylates STAT3 [17]. Others have reported that the MAP kinase phosphatase MKP-1 can dephosphorylate angiotensin-II-activated STAT1, but not STAT3 [10,11]. Thus MKP-1 might be a suitable candidate to mediate the selective suppression by IL-1$\beta$ of the activation of STAT1, but not that of STAT3, by IL-6. Other recent evidence suggests that a nuclear PTP is required for the inactivation of STAT1 [12]. Whether this nuclear tyrosine phosphatase (as yet undefined) or MKP-1 is responsible for the suppression of IL-6-activated STAT1 by IL-1$\beta$ requires further investigation.

In summary, we have demonstrated for the first time that IL-1 inhibits IL-6-induced STAT1 tyrosine phosphorylation by a proteasome-dependent mechanism, followed by the down-regulation of IL-6-induced STAT activation. Although we could not provide conclusive evidence that the inhibition by IL-1 of STAT1 phosphorylation is involved in the suppression by IL-1 of IL-6-induced type II acute-phase proteins, two lines of evidence support this notion. First, it has been reported that STAT1 and STAT3 can bind to the promoters of several type II acute-phase proteins, including $\alpha_2$-macroglobulin and $\gamma$ fibrinogen [42]. Secondly, overexpression of dominant-negative STAT1 mutants markedly inhibited the IL-6-induced expression of human $\gamma$ fibrinogen mRNA (X. Shen, Z. Tian and B. Gao, unpublished work), suggesting that the activation of STAT1 is involved. It has been reported that IL-1$\beta$ completely abolished IL-6-induced the expression of type II acute-phase genes ([3], and X. Shen, Z. Tian and B. Gao, unpublished work), whereas IL-1$\beta$ only partly suppressed IL-6-activated STAT (it inhibited only STAT1, not STAT3), suggesting that the attenuation of STAT1 phosphorylation is only one of the mechanisms involved in the suppression by IL-1$\beta$ of IL-6-induced type II acute-phase gene expression and that other mechanisms might also be involved. It has been reported that both $\alpha_2$-macroglobulin promoter and $\gamma$ fibrinogen promoter contain two STAT3/NF-$\kappa$B overlapping binding sites and that the activation of NF-$\kappa$B by IL-1$\beta$ can suppress STAT3/STAT1 binding [42,43]. The probe m67 used here does not contain an NF-$\kappa$B-binding site; no apparent binding of NF-$\kappa$B to m67 was observed after stimulation with IL-1$\beta$. Therefore at least two mechanisms are involved in the suppression by IL-1$\beta$ of IL-6-induced type II acute-phase gene expression: (1) the dephosphorylation of STAT1 by a proteasome-dependent mechanism, followed by the down-regulation of IL-6-induced STAT activation; (2) the attenuation of STAT1/STAT3 binding to acute-phase gene promoters by activated NF-$\kappa$B [43]. Additional studies will be required to define more precisely how proteasome-dependent mechanisms are involved in the suppression of IL-6-activated STAT1 by IL-1$\beta$ but the findings already provide a useful model for cross-talk between the IL-1$\beta$ and IL-6 signal-transduction pathways. In particular, the findings provide an initial explanation for how IL-1$\beta$ concomitantly induces NF-$\kappa$B while down-regulating STAT-dependent pathways in hepatic cells. This type of combinatorial action might therefore be crucial for the IL-1-dependent selection of gene expression during the acute-phase response to tissue injury.

### Note added in proof (received 2 November 2000)

Since this paper was originally submitted, Ahmed and Ivashkiv [44] reported that IL-1$\beta$ inhibited IL-6 signalling in primary macrophages. These results are in agreement with ours and

suggest that inhibition of IL-6 signalling by IL-1$\beta$ is not cell-type-specific.

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